Influence of N-methyl pyrrolidone on the activity of the pulp-dentine complex and bone integrity during osteoporosis

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Abstract: AIM To analyze the effect of systemic application of N-methyl pyrrolidone (NMP) on the pulp-dentine complex and on the jawbone of ovariectomized rats. METHOD Female Sprague-Dawley rats were randomly divided into a sham-operated group (Sham n=6) and an estrogen depletion by ovariectomy (OVX n=12) group. In 6 of the ovariectomized animals N-methyl pyrrolidone (NMP) in phosphate-buffered saline (PBS) was administered systemically weekly by intraperitoneal injection (i.p.); the other 6 were injected with PBS (Veh). After 15 weeks of injections the jaw bones were collected and pulps extracted from the incisors teeth. Histology was used to determine pre-dentine thickness in teeth and radiography to determine alveolar bone mass. Immunohistological staining and RT-PCR were performed to verify the presence and localization of the odontoblast specific dentine sialoprotein and to quantify its expression in the dentine pulp complex. Mandibular cortical width and mandibular height was evaluated by means of X-ray analysis. Statistical analysis was performed with analysis of variance (ANOVA). RESULTS Both pre-dentine (P=0.029) and alveolar bone structures (P=0.049) were significantly reduced due to estrogen deficiency in OVX Veh and OVX NMP treatment normalized these parameters to the Sham level. DSPP expression in OVX NMP animals was significantly higher (P=0.046) than in OVX Veh. X-ray analysis confirmed that ovariectomy significantly reduced the mandibular cortical width in the OVX Veh group compared to the Sham Veh and OVX NMP (P=0.020). CONCLUSION N-methyl pyrrolidone (NMP) had a remarkable anti-osteoporotic ability preserving the activity in the pulp-dentine complex and preventing jawbone loss. These effects make NMP a promising candidate for the preservation of the activity of the pulp-dentine complex and the jawbone thickness in postmenopausal females. This article is protected by copyright. All rights reserved.

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Influence of N-methyl pyrrolidone on the activity of the pulp-dentine complex and bone integrity during osteoporosis

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Running head: NMP preserves pulp-dentine activity

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Introduction

Osteoporosis is a metabolic disease characterized by low bone mass, where the deterioration of trabecular bone architecture is significantly affected, leading to greater probability of bone fracture. Some of the major risk factors that lead to osteoporosis include: age, estrogen deficiency, mineral insufficiency or immobilization (Diab et al. 2006, Lacativa et al. 2006, Reginster et al. 2006).

Recently it was reported that osteoporosis not only affects the skeleton but also reduces the dentinogenic capacity of rat mandibularincisors and the activity of the pulp-dentine complex (Xu et al. 2014). Moreover osteoporosis caused by estrogen deficiency is associated with periodontal bone loss, reduced jawbone and tooth loss (Ames et al. 2010, Mijares et al. 2012a).

There have already been a great deal of studies using the ovariectomized rat model as the golden standard in examining osteoporotic changes predominantly in bone structure and more specifically in jawbones (Yang et al. 2003, Yang et al. 2005, Rowshan et al. 2010). Also, it has previously been described how estrogen deficiency did indeed cause a significant decrease in bone mass in the alveolar interradicular septa of adult rat first molars (Tanaka et al. 2002).

It wasalready shown that N-methyl pyrrolidone (NMP), a drug vehicle and solvent used as a constituent in FDA-approved medical devices plays a significant role in the osteoblast and osteoclast differentiation (Miguel et al. 2009, Ghayor et al. 2011). Indeed, NMP enhances BMP-2-induced osteoblast differentiation and bone regeneration and disrupts osteoclast differentiation and bone resorption. In dentistry, NMP has been used as a constituent in guided bone regeneration membranes, guided tissue regeneration membranes, and bone substitute materials (Southard et al. 1998, Zwahlen et al. 2009, Schmidlin et al. 2013, Schneider et al. 2014). NMP is also a functional low affinity acetyl lysine mimetic and Bromodomain inhibitor with pleotropic anti-myeloma and immunomodulatory activities (Shortt et al. 2014).

A high affinity Bromodomain inhibitor, JQ1, was recently shown to prevent bone destruction in a periodontitis model (Meng et al. 2014). Only recently, it wasdemonstrated that NMP preserved both mass and quality of long bones in ovariectomized rats while its effect on non-ovariectomized control animal group was not significant changed (Gjoksi et al. 2015) . These results question whether NMP had a similar protective effect in the jawbone and teeth of an osteoporosis (estrogen depletion by ovariectomy) animal model. The aim of this study was to analyze the effect of systemic application of NMP on the pulp-dentine complex and on the jawbone of OVX rats.

Material and Methods

Osteoporosis rat model and treatment

Experiments with animals were carried out in accordance with the Guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations (Veterinäramt Kanton Zurich; 40/2012). Sprague-Dawley (SD) female rats (wt. 230 ± 10 g/ 15 week old), (mean ± SD) , were obtained from Charles River laboratories and were adapted to laboratory environment for 2 week before surgery. The acclimatized animals underwent ovariectomy or sham operation in accordance with previously established techniques (Kharode et al. 2008) (Sham, n=6) or bilateral ovariectomy (OVX, n=12). After recovering from surgery, the ovariectomized rats were divided into 2 groups: OVX with PBS as vehicle (OVX-Veh, n=6) and OVX with NMP (OVX-NMP, 1/3 of LD50 = 105µL/100g/week, n=6). One animal from the OVX Veh group died in the second week of unknown reasons. Treatment of ~8mM NMP (NMP/PBS mix) or PBS was initiated 1 week after OVX and lasted for 15 weeks. 700 µL PBS or 105 µL NMP/100 g weight of the animal made up to 700 µL with PBS were injected i.p. once a week. The body mass of each animal was monitored weekly, and the administered dose was adjusted accordingly. All animal procedures were approved by the Zürich University Animal Ethics Committee (approval 40/2012) and meet the ARRIVE Guidelines (PMID: 20613859).

After 15 weeks, the rats were sacrificed by CO_2 inhalation, employing a CO_2 chamber (Euthanex, Palmer, PA 18043 USA). The jawbones were separated, cleaned of soft tissue, and stored in 70% alcohol. The mandibles, cleaned of extraneous tissue, were subjected to 1% enzyme treatment (Terg-A-Zyme, Alconox Inc, New York, USA) at 50 °C for 48 h. The hemi-mandibles were separated at the symphysis, incisors were removed and then stored in 70% alcohol. Radiographs of the hemi- mandibles and incisors were taken with CCX digital (computer controled x-ray timer); Trophy Irix 70 (Jordi Röntgentechnik AG, Münchenstein, Switzerland). Whole blood samples were collected via abdominal aorta puncture immediately following sacrifice. Then, a serum specimen was harvested after centrifugation (2000 rpm for 20 analyzed via an Enzyme-linked Immunosorbent Assay (ELISA) from (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) in accordance to the recommendation with the manufacturer. Statistical analyses were performed using SPSS statistics 22 to test the mean difference (One-way ANOVA) between groups at type- 1 error 0.05

Histology and predentine thickness measurement

Paraffin sections for histological analysis of bone quality were prepared and stained with haematoxylin and eosin (H&E). The sections were examined for changes in the alveolar process and staining was visualized with a Leitz Dialux20 microscope and images were captured using a Leica camera. Ten randomized areas of the predentine were selected from H&E stained paraffin sections of the mandibularincisor of each animal. Mirax viewer 1.1 (3DHISTECH Ltd. Budapest, Hungary) was used to identify the area and measure the thickness of predentin.

Immunohistochemistry

Immunohistochemical localizations of dentine sialoprotein (DSP) were observed in additional serial sections of the specimens. Histologic slides were prepared as previously described

(Ruangsawasdi et al. 2014) and incubated with mouse/anti-rat primary antibody (Merck Millipore, Darmstadt,Germany) against DSP (1:100) at room temperature for 5 h. 3,3' – diaminobenzidine tetrahydrochloride (DAB) was used. Samples were then incubated with rabbit/anti-mouse secondary antibody (Merck Millipore) for the detection of the immunoreactivity of the DSP marker. For all immunohistochemical analysis negative controls were used. Slides were counterstained with haematoxylin and Eosin and observed by light microscopy.

RNA isolation from pulp tissue

RNA was isolated from maxillary incisor tooth pulp tissue of all animals and conserved by immediate freezing in liquid nitrogen. The frozen pulp was homogenized separately in 1.5mL PCR grade tube containing 1ml TRIZOL (life technology, Zug, Switzerland) at 4°C using a homogenizer (Omni international, Kennesaw, USA) and were left to solubilize in the TRIZOL for 5 min. Subsequently, 200 µL of chloroform (Sigma-Aldrich, Buchs, Switzerland) was added and the specimens were shaken vigorously for 15 sc followed by 5 min incubation at room temperature. The tubes were then centrifuged for 15 min, 14800 gat 4°C (centrifuge 5417R, Eppendorf, Hambourg, Germany) to yield a three layered solution. The RNA phase was transferred to a new tube and precipitated with an equal volume of isopropanol for 10 min at room temperature. Centrifugation (14800 g at 4°C) for 15 min was again performed to collect the RNA pellet. Supernatant was discarded and 1mL of 75% (v/v) ethanol was added to wash the pellet followed by centrifugation (7500 g for 5 min).

The ethanol was discarded before RNA purification with RNeasy mini kit (Qiagen, Hilden, Germany). The quantity and quality of the RNA was checked before storage at -20°C by Nano drop and 1% agarose gel.

RT-PCR

Two step RT-PCR was used for RNA quantification. 200 ng of total isolated RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (Roche, Rotkreuz, Switzerland). The master mix was prepared following the manufacturer's instruction.

Real-time PCR was performed using the LighCycler® 480 DNA SYBR Green I master (Roche). The rat-specific forward (F) and reverse (R) DSPP primers (R:5`CGTTGCTGTCTTTACTT CCACT3`, F:5`ACACAGGACAACCAGAATCTCA3`), were used in this experiment, while GAPDH (R:5`GGATGCAGGGATGATGTTCT3`, F:5`GAAGGGCTCATGACCA CAGT3`) was the internal control. Relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method as previously described (Livak et al. 2001).

Statistical analysis was done with One-way ANOVA and Post hoc comparisons using the Fisher LSD test ($P<0.05$). The box plot shows median and whiskers.

Morphometric analysis of the mandibular bone

Mandibular cortical width (MCW), a measurement related to the resorption of the mandible cortical bone (Mijares et al. 2012b), was determined by drawing a line parallel to the long axis of the mandible and tangential to its inferior border. Another line was drawn parallel to the first line up to the width of the inferior cortex. The perpendicular distance between these two lines was recorded by ImageJ (open source; http://imagej.nih.gov/ij/). Three different measurements were made in this region and the mean of the values reflected the MCW. Mandible height was calculated by measuring the distance between the mandibular foramen and the inferior mandibular cortex by ImageJ, a measure of the degree of the alveolar crest resorption. Statistical analyses were performed using SPSS statistics 22 (IBM, Armonk, North Castle, USA) to test the mean difference (One-way ANOVA) between groups at type- 1 error 0.05.

Statistical analysis

All statistical analyses were performed with IBM SPSS statistics 22. Data from all parameters are normally distributed (Shapiro–Wilk test) unless otherwise stated. Results are expressed as the mean ± SD and are compared by ANOVA and Student's *t*-test. Results are considered significantly different for $p<0.05$.

Results

Effect of NMP on animal body weight and estradiol level

Over the 15 week period the OVX Veh group (n=5) gained on average significantly (P=0.001) more weight (155 ± 9 g) than the Sham PBS group (90 ± 13 g) or the OVX NMP group (n=6) (95 ± 24 g). The estradiol level, however, was significantly (P=0.004) higher in the Sham PBS (n=6) group (209 ± 7 pg/ml) than in the OVX Veh (144 ± 25 pg/ml) or the OVX NMP group (168 ± 26 pg/ml). These parameters indicate the successful establishment of the estrogen deficient animal model needed for the study.

Variation of predentine thickness in rat incisors

Histological assays revealed that the predentine structure in Sham Veh incisors (Fig.1A) was significantly thicker (p value=0.029) than those in OVX Veh incisors. Moreover, when ovariectomized animals were treated with NMP the predentin structure was thicker p= 0.020 than the non-treated group (OVX Veh). In the incisors of the OVX NMP (n=6) group predentine thickness appeared similar to that in the Sham Veh group and significantly higher p=0.020 than in the OVX Veh group (Fig 1B).

Changes in pulp-dentine complex markers at the protein and RNA level

Immunohistochemical staining of ovariectomized rats (OVX Veh) displayed a reduction in DSP immunoreactivity (brown stained layer/odontoblastic layer) while DSPP expression was stronger in Sham Veh (n=6) and OVX NMP (n=6) incisors (Fig.2A). RT-PCR assay further confirmed what was observed from the immunohistochemical staining this time using DSPP

as a marker. mRNA expression of DSPP was significantly weaker(p=0.046) in OVX Veh group as compared with OVX NMP (OVX Veh vs. OVX NMP ¶ (P<0.05).
However, when the DSPP gene expression was compared between OVX Veh (n=5) and Sham Veh the expression differences were not significant (Fig.2B).

Crown and jawbone morphology

Changes in crown morphology between the different groups were observed. The clinical crowns of the mandibular incisors of the OVX Veh (n=5) group were shorter than the Sham (n=6) and OVX NMP (n=6) group as shown in the Figure 3A. X-rays of the lower semi mandible reveals slight morphological differences between the groups such as the curvature of the incisor and the curvature of the mandibular body. The latter appears to be at a narrower angle in ovariectomized animals compared to Sham (Figure 3B). In the incisor radiographs, the pulp-dentine complexes of OVX (n=5) and SHAM rats (n=6) did not generally differ.

Morphometric analysis of the mandibular bone

Morphometric measurements based on the radiographs of the jawbone were used to quantify the degree of bone resorption: (1) the mandibular cortical width (MCW), a measure of the reduction in cortical bone; (2) mandible height corresponding to the total height of the mandible (Fig 4 A, B). These two parameters were used to measure any change in jawbone which occured due to osteoporotic bone resorption. The mandibular cortical width (Fig.4A) was significantly different p=0.006 between Sham (n=6) and OVX Veh (n=5), but in the OVX group treated with NMP (n=6) we saw a significant increase p=0.049 in MCW reaching parameters very similar to the Sham group. (Sham Veh vs. OVX Veh * (P<0.05) and OVX Veh vs. OVX NMP ¶ (P<0.05)). Mandible height on the other hand even though the difference and trend between groups was similar to that of the MCW, was not significantly altered due to the large variationbetween animals in the same group (Fig 4B).

Histology of the alveolar process

Static histomorphometry on hemimandible samples was performed with haematoxylin and eosin (H&E). Bone in the alveolar process region, under the roots of the molars was analyzed and compared between different animal groups. The results support what was already observed by the morphometric analysis which show that NMP treatment prevented bone loss in the OVX-treated group compared to the control group (Fig 5).

Discussion

N-methyl pyrrolidone (NMP), a FDA-approved small molecule has been applied in the dental field within guided bone and tissue regeneration membranes as well as bone substitute materials (Southard et al. 1998, Zwahlen et al. 2009, Schmidlin et al. 2013, Schneider et al. 2014). It has been proven that NMP prevents long bone loss in OVX rats while no effect is observed in non-ovariectomized animals (Gjoksi et al. 2015). The aim of the present study wasto evaluate its effect on dentinogenic activity and resorption of alveolar bone in a compromised situation mimicking osteoporosis in menopaused women.

Monitoring the weight and determining the estradiol level after 15 weeks showed that the ovariectomized animals were indeed estrogen deficient. Since, the estradiol level in the group treated for 15 weeks with (OVX NMP) did not show any significant difference to that of the OVX Veh group, this suggests that the effect of NMP is not based on changes in estrogen levels but rather on the bioactivity of NMP (Miguel et al. 2009, Ghayor et al. 2011, Gjoksi et al. 2015)

Earlier studies have demonstrated that predentine can provide a supporting mechanical function for the pulp tissue (Xu et al. 2014). Additionally, the predentine layer thickness directly correlates with the dentinogenic activity function in human teeth (Couve 1987). The results demonstrated that the predentine layer was significantly thinner in the incisors of theOVX Veh group compared to the incisors of the Sham Veh group establishing that dentinogenic activity is inhibited after ovariectomy. Xu et al., (Xu et al. 2014) linked their finding on the molecular level to a decrease in Runx2 and osterix expression. In the present

study NMP preserved the predentine structure, since in the OVX NMP group predentin was significantly thicker than in the OVX Veh group (Fig.1A). This might be due to the induction of osterix by NMP via the enhancement of the kinase activity of the BMP-BMP-receptor complex for Smad and p38 as was shown previously (Miguel et al. 2009). Runx2 will certainly not be induced by NMP, since its affinity to BRD4 (Gjoksi et al. 2015) will prevent BRD4 from its essential contribution in forming an active Runx2 promotor complex as was described for osteoblast differentiation (Lamoureux et al. 2014).

Estradiol deficiency has been shown to also inhibit the proliferation and differentiation of dental pulp stem cells (DPSC) (Zhang et al. 2011). Expression of DSPP and presence of DSP are usually used to monitor DPSC as they are largely used for studies of odontoblast differentiation (Liu et al. 2005). In the present study, the OVX Veh group had the lowest DSP expression level (odonto/osteogenic protein) in the pulp-dentine complex as seen on the immunohistological staining compared to Sham Veh incisors. These findings indicate that estrogen deficiency down-regulated dentinogenesis leading to a decline in mineralization (d'Aquino et al. 2009, Xu et al. 2014). Furthermore, in the NMP treated group (OVX NMP), DSP expression was clearly preserved and had a similar staining intensity to the Sham Veh group (Fig. 2A). Similar effects were observed at the mRNA level where DSPP expression was much lower for OVX Veh compared to Sham and OVX NMP (Fig. 2B). Since NMP had no effect on estrogen levels, these results suggest that NMP treatment prevented dentine activity deterioration via an estrogen independent route.

Activation of NF-kB pathway can promote the odontoblastic phenotype and stimulate odonto/osteogenic differentiation of dental pulp-derived stem cells in an estrogen deficiency rat model (Paula-Silva et al. 2009, Yang et al. 2012, Wang et al. 2013). The NF-kB pathway as mechanism for the effect of NMP on restoring the dentinogenic activity can be excluded, since NMP will prevent binding of BRD4 to acetylated lysine-310 of RelA, which is a NF-kB subunit and therefore essential for the recruitment of BRD4 to the promoters of NF-κB target genes and to coactivate NF-κB (Huang et al. 2009). Therefore more intensive studies are required to explore the mechanism of action of NMP on odontoblast activity.

In humans mandibular radiomorphometric indices are sometimes used to diagnose osteoporosis. It has been shown that long term estrogen deficiency in ovariectomized rats decreases the mandibular cortical thickness (Yang et al. 2005). These mandibular cortical indices have been developed to allow quantification of mandibular bone mass (Devlin et al. 2002). Similar indices such as mandibular cortical width (MCW) and total mandibular height to measure bone mass in the mandibles we used in the OVX animal model of the present study.

The MCW of the OVX Veh group was significantly smaller than in the Sham Veh group. This confirms that estrogen deficiency induced osteoporosis leads to resorption of the mandible. Conversely, NMP treatment significantly prevents bone loss of the mandible as demonstrated by the mandibular cortical width preservation in the OVX NMP animal group (Fig. 4A). This is in line with the inhibition of osteoclast maturation and activity by NMP via blocking ERK phosphorylation known to reduces cFos transcription and AP-1 activation needed for the induction of NFATC1 transcription which is central for osteoclast formation (Ghayor et al. 2011). Another route to induce NFATC1 transcription is via RANKL activation. Bromodomain inhibitors like JQ1 block RANKL activation by binding BRD4 and preventing it from binding to the RANKL promotor region complex and inducing its transcription (Lamoureux et al., 2014).

Similar to JQ1, NMP was found to be an epigenetically active substance although with low affinity for bromodomains in BRD4 (Gjoksi et al. 2015). Due to its dual activity, NMP blocks both routes of NFATC1 transcription initiation and appears very effective in the suppression of bone destruction in the mandible.

Conclusion

Long-term estrogen deficiency as manifested in osteoporosis resulted in impaired dentinogenic regeneration capacity and mineralization whereas, NMP treatment led to a

prevention of such phenomenon. The presentstudy provides evidence that NMP, a widely used drug vehicle and constituent in several medical devices used in dentistry, may have potential as a drug for treating postmenopausal females to preserve the activity of the pulpdentine complex and to prevent alveolar bone loss. Further studies, with larger numbers of subjects, are warranted in order to better elucidate the potential of NMP treatment for preservation of the activity of the pulp-dentine complex and to prevent alveolar bone loss in postmenopausal females.

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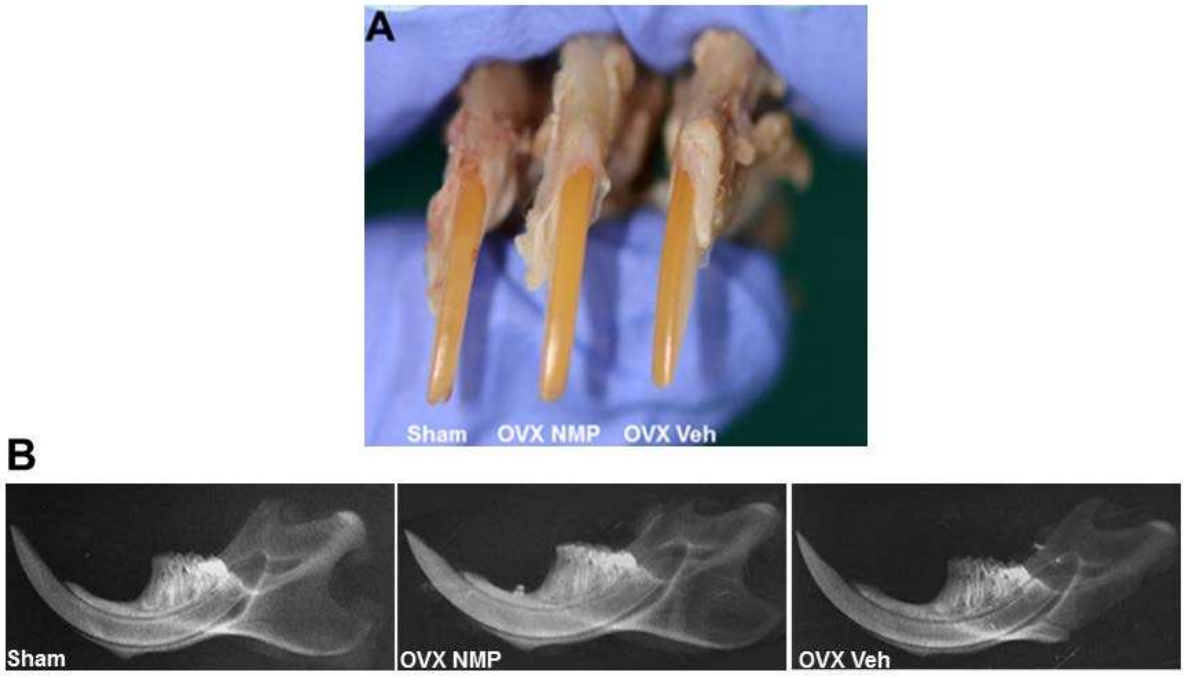

Figure 1: Predentine thickness changes in OVX rat incisors. (A) The analysis of histological sections from incisors stained with H&E demonstrated that predentine seen as a white/light pink layer (marked by arrows) was significantly thicker in Sham Veh incisors compared to OVX Veh incisors. (B) In the incisors of the OVX NMP group predentin appeared similar to that in the Sham Veh group and statistically higher than in the OVX Veh group Sham Veh vs. OVX Veh * ($P=0.004$) and OVX Veh vs. OVX NMP ¶ ($P<0.05$). Grey bars and error bars indicate means ± SD.

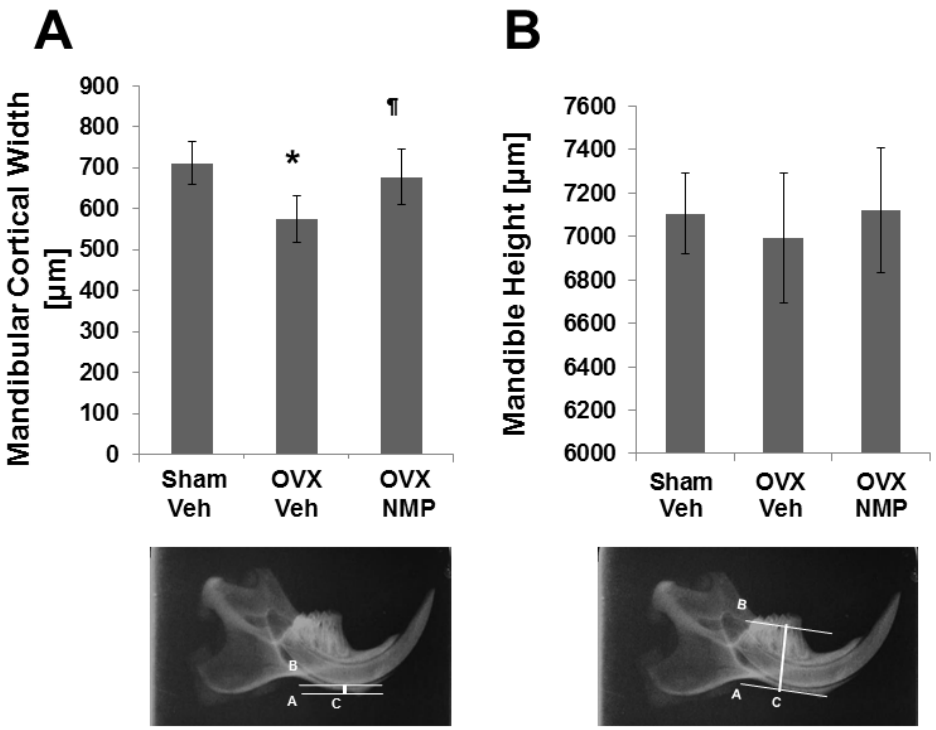

Figure 2: Changes of dentin markers after ovariectomy. (A) Immunohistochemical staining of DSP in paraffin sections showed that DSP (brown color) was more intense in Sham Veh and OVX NMP animals than in OVX Veh animals. (B) This observation was confirmed by RT-PCR assay of DSPP expression in the three groups. In OVX NMP animals, expression of DSPP was significantly higher than in OVX Veh animals and surpassed even the DSPP expression in Sham Veh animals, (OVX Veh vs. OVX NMP ¶ ($P<0.05$). Statistical analysis was done with One-way ANOVA and Post hoc comparisons using the Fisher LSD test ($P<0.05$). The box plot shows median and whiskers.

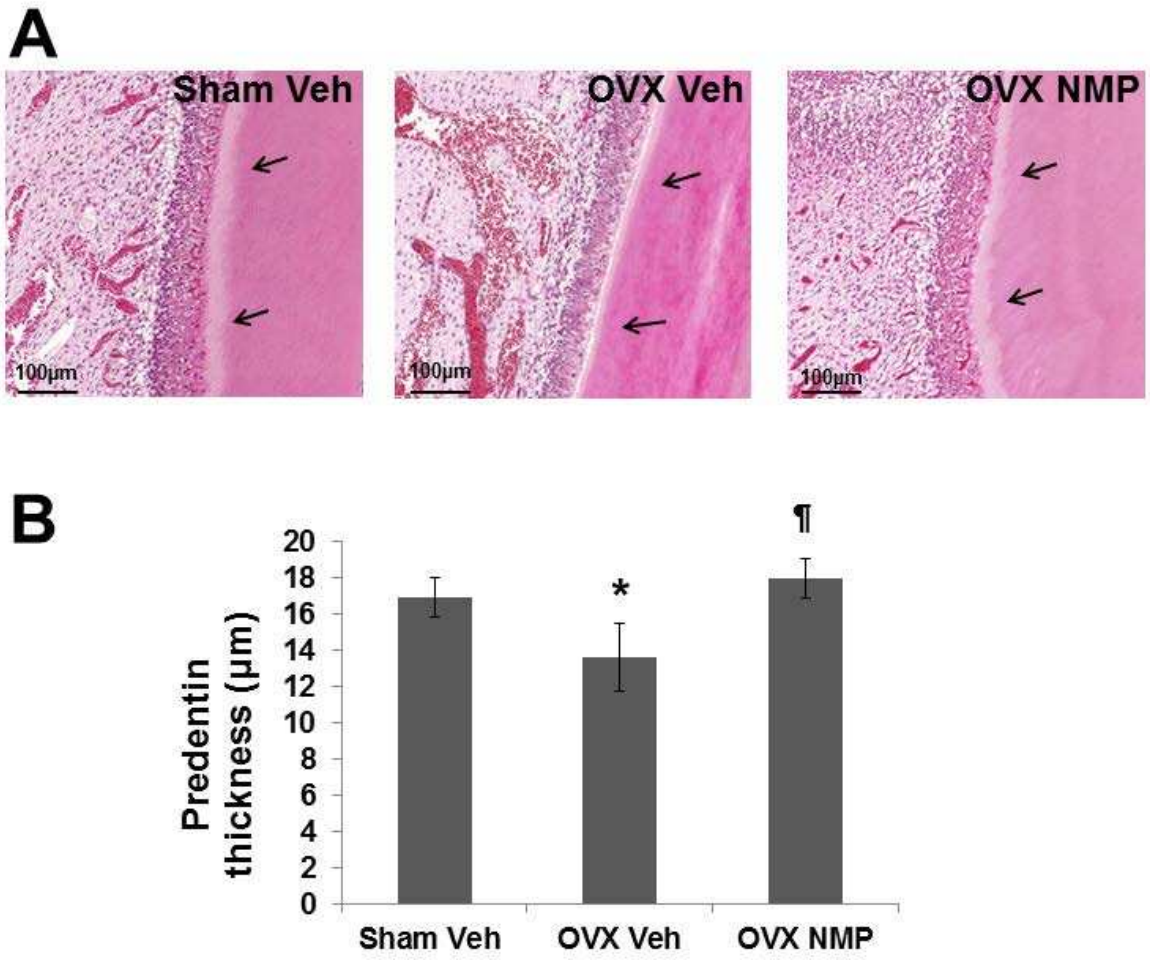

Figure 3: Morphology of incisors and lower semi mandible were observed after sacrifice. Incisors crown shape after ovariectomy (OVX) has changed and appears shorter (A). X-rays of the lower semi mandible show slight morphology differences between the groups like the curvature of the incisor. The curvature of the mandibular body appears to be at a narrower angle in ovariectomized animals compared to Sham (B).

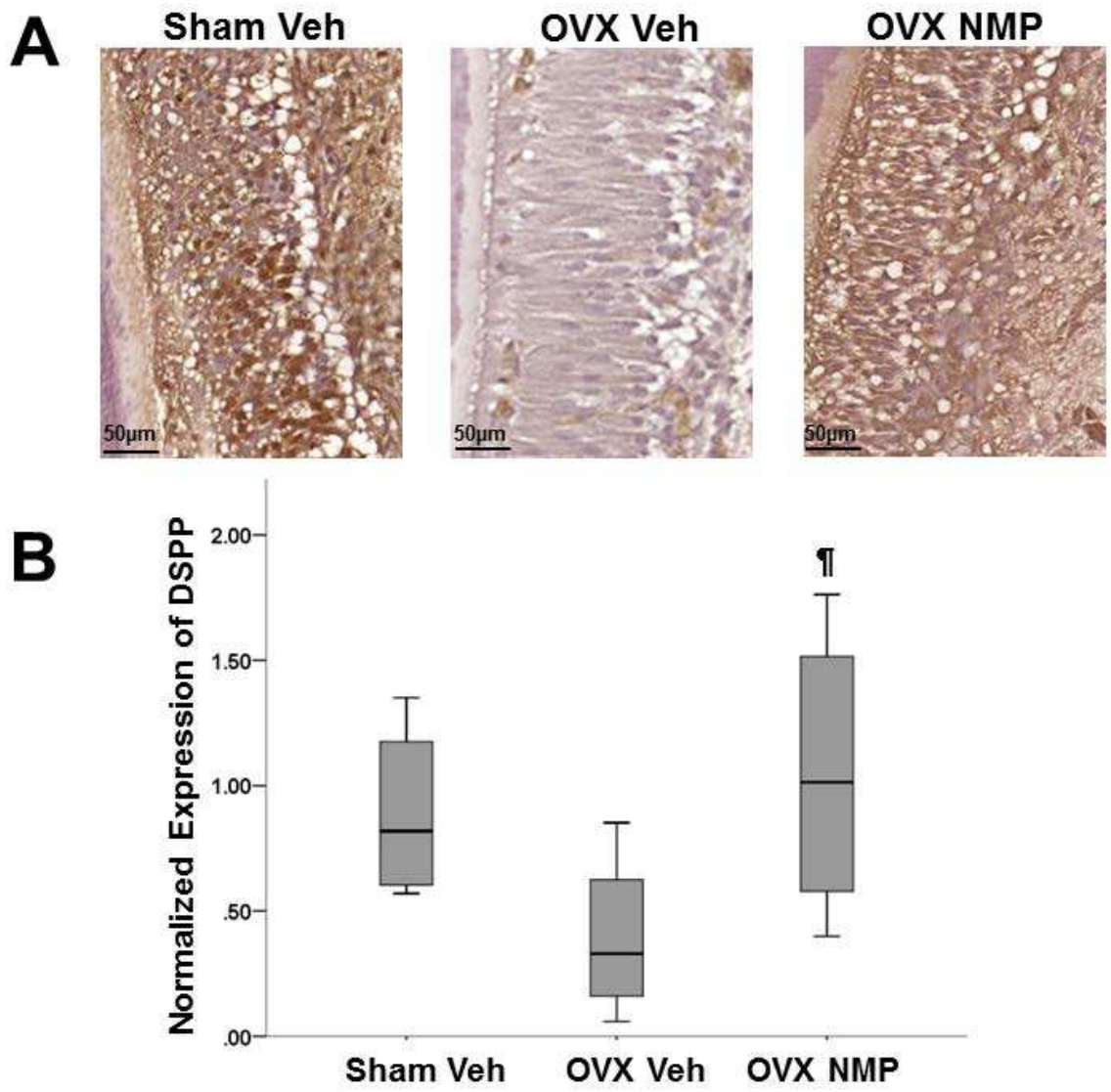

Figure 4: Morphometric analysis based on radiographs of the mandible. (A) The mandibular cortical width (MCW) was measured to assess the reduction in cortical bone. MCW was measured as the distance (line C) between two tangents to the inner (line I) and outer (line O) border of the cortical bone, which were aligned parallel to the long axis of the mandible (lower panel). In figure 2B the total mandible height (D) was measured as the distance between the mandibular foramen (MF) and the inferior mandibular margin (IMM) (lower panel). A comparison of these two parameters in the three groups showed that the MCW was significantly greater in Sham Veh and OVX NMP animals than in OVX Veh animals (Sham Veh vs. OVX Veh * ($P<0.05$) and OVX Veh vs. OVX NMP ¶ ($P<0.05$)). Mandible height displayed a similar tendency (B) but the differences between groups were not significant. Grey bars and error bars indicate means ± SD.

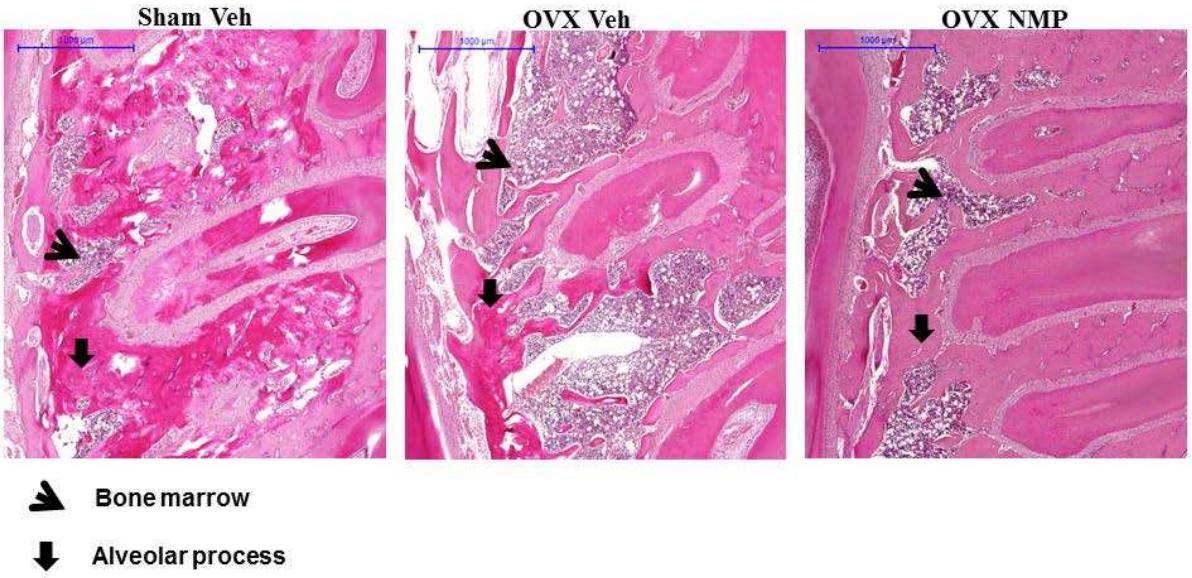

Figure 5: Static histomorphometry of the alveolar process. Mandible paraffin sections from rats were stained with hematoxylin and eosin to visualize and compare alveolar bone surfaces between the groups. Sections stained with H&E show a clear indication of denser trabecular bone (pink) in both Sham and OVX NMP treated group compared to OVX Veh. Bone marrow seen in purple (arrow) is much more extensive in the OVX Veh group compared to both Sham Veh and OVX NMP.